Biology of the Triple-Negative Breast Cancer: Immunohistochemical, RNA, and DNA Features

Mercedes Herrera Juarez · Pablo Tolosa Ortega · Ana Sanchez de Torre
Eva Ciruelos Gil

Division of Gyneco-Oncology, Breast Cancer Unit, University Hospital 12 de Octubre, Madrid, Spain

Keywords

Triple-negative breast cancer · Intrinsic subtypes · Biomarkers · Immuno-oncology

Abstract

Background: The triple-negative breast cancer (TNBC) constitutes a heterogeneous disease with an aggressive behavior and a poor prognosis. A better understanding of its biology is required to identify new biomarkers and improve clinical outcomes. **Summary:** To date, the definition and classification of TNBC depends on a multiomic approach including immunohistochemistry (IHC), genomic, and transcriptomic features, and the tumor immune landscape. The development of new technologies has allowed us to sequence the whole cancer genome. The Cancer Genome Atlas (TCGA) and next-generation sequencing have led to a greater knowledge of DNA alterations such as TP53 or BRCA mutations, copy number variations, and DNA methylations. In addition, gene expression profiling has allowed to define a molecular intrinsic classification of TNBC based on mRNA. IHC and genomic profiling are also necessary to identify new immune biomarkers such as the presence of tumor-infiltrating lymphocytes and the expression of immune checkpoint molecules. **Key Messages:** This review aimed to provide recent knowledge of TNBC biology and classification focused on IHC, transcriptomics, genomic features, and the new immune biomarkers.

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Introduction

Triple-negative breast cancer (TNBC) is a heterogeneous disease with a poor prognosis, defined by the absence of estrogen receptor (ER), progesterone receptor (PR), and HER2 protein overexpression [1]. TNBC represents 10–15% of all breast tumors and is characterized by poorer survival, the lack of solid biomarkers, and absence of personalized targeted therapy.

TNBC patients are at high risk of relapse in the first 5 years, especially if a pathological complete response (pCR) has not been obtained [2], despite the use of conventional chemotherapy based on anthracycline and taxane regimens, the addition of platinum agents, and the use of adjuvant capecitabine [3]. It is known that stage I tumors with stromal tumor-infiltrating lymphocytes (TILs) >30% have an excellent prognosis without adjuvant treatment [4]. However, those patients who relapse early or progress during neoadjuvant chemotherapy (NACT) represent a real unmet medical need.

Beyond the classical definition of TNBC, we should analyze TNBC with a multiomic approach including immunohistochemistry (IHC), transcriptomics, genomics, and new immune biomarkers. Breast cancer intrinsic subtypes play an essential role to classify these tumors. We know that within TNBC all intrinsic subtypes can be identified. The aim of this review is to provide a multiomic approach to TNBC.

Histological and IHC Approach

We can dissect TNBC by morphological and IHC features. Several approaches such as histological grade or the presence of favorable histology (medullary breast carcinoma or adenoid cystic carcinoma) or aggressive histology (metaplastic carcinomas or sarcomatous carcinomas) could guide our initial decision. The IHC analysis is essential in TNBC definition, as well as the Ki-67 proliferation index, the presence of androgen receptor (AR) and basal cytokeratins, and programmed death-ligand 1 (PD-L1) expression.

Ki-67

To evaluate cell proliferation rates, Ki-67 expression is the most used cell membrane antigen and is considered a prognostic biomarker in breast cancer (BC). Ki-67 expression >20% is found in up to 50% of TNBC and used to be higher than in luminal BC [5]. Recently, a retrospective study showed that higher Ki-67 expression is an independent prognostic factor in disease-free survival (DFS; RR 2.83, 95% CI: 1.58–5.06, $p < 0.001$) and OS (RR 3.18, 95% CI: 1.48–6.79, $p = 0.003$) [6], and it is significantly correlated with the TNBC phenotype [7]. After NACT, patients with Ki-67 expression increasing $\geq 20\%$ had a worse DFS than patients with stable or decreased Ki-67 expression ($p < 0.001$) [8].

Androgen Receptor

AR expression is another important marker. AR expression in TNBC may vary widely between 10 and 90% according to the cohort and the cutoff used for AR positivity (≥ 1 or $>10\%$) [9, 10]. Within non-basal-like TNBC, AR could be a predictive response biomarker. In TNBC cell lines with positive AR expression, AR stimulation results in growth activation while AR antagonists promote growth decline [11]. Recently, 2 phase II trials have been published demonstrating the activity of abiraterone acetate plus prednisone and enzalutamide in AR-positive TNBC patients [12, 13]. Despite these findings, the development of antiandrogens in this subset has not been correctly established due to the absence of a homogeneous definition for AR positivity and the low rate of AR positivity withing TNBC. However, interest in this field is growing, and new trials are guaranteed.

Epidermal Growth Factor Receptor

The epidermal growth factor receptor (EGFR) plays an important role in cell proliferation and apoptosis inhibition. EGFR overexpression in TNBC is variable among studies ranging from 13 to 78% [14, 15]. Data from EGFR protein overexpression in triple-negative cancer is controversial and has not been confirmed as a prognostic bio-

marker. Targeting this pathway by tyrosine kinase inhibitors, such as gefitinib, afatinib, and erlotinib, or by monoclonal antibodies such as cetuximab and panitumumab [16, 17], has not demonstrated any sign of clinical activity.

Basal Cytokeratins

Basal-like BC represents 75% of TNBC and is considered an aggressive subtype defined classically by basal cytokeratins: CK 5/6, CK 14, and CK 17 [18]. CK 5/6 expression ranges from 24 to 72% in TNBC [19, 20], CK 14 and CK 17 overlaps with CK 5/6. Some results suggested that CK 5/6-positive TNBC have poorer prognosis; despite that, the CIBOMA trial showed that a nonbasal phenotype according to CK 5/6 and/or EGFR expression by IHC resulted in a significantly increased DFS (HR, 0.53; 95% CI, 0.31–0.91; $p = 0.022$) and overall survival (OS) (HR, 0.42; 95% CI, 0.21–0.81; $p = 0.0095$) when capecitabine therapy was used [21].

DNA: Mutation, Copy Number Variation, and Methylation

The Cancer Genome Atlas (TCGA) analyzed primary BC by genomic DNA copy number arrays, DNA methylation, exome sequencing, messenger RNA arrays, microRNA sequencing, and reverse-phase protein arrays [22]. Despite the large number of genes traditionally mutated in BC (PIK3CA, PTEN, AKT1, TP53, GATA3, CDH1, RB1, MLL3, MAP3K1, and CDKN1B) and novel significantly mutated genes (TBX3, RUNX1, CBFB, AFF2, PIK3R1, PTPN22, PTPRD, NF1, SF3B1, and CCND3) and the high tumor mutational burden (TMB), the diversity and recurrence of mutated genes in TNBC is lower than in luminal subtypes. TP53 mutations occur in 80% of TNBC followed by PIK3CA mutations (9%), while other driver mutations are quite infrequent.

TP53

TP53 mutations in TNBC were mostly nonsense or frame shift mutations, and they occur in 65–80% of cases [23]. p53 protein expression in TNBC tumor tissue changes according to the type of mutation occurring in the TP53 gene: missense mutations tend to show high p53 protein expression and increased protein stability, which is in contrast with deletion mutations that lead to a lack of protein expression. Mutant p53 cannot be recommended as a prognostic or therapy-predictive biomarker in BC and has been considered as *undruggable*. Despite that, several compounds have become available, and they can reactivate mutant p53 protein and convert it to a conformation with wild-type properties. Some of these compounds, especially PRIMA-1, PRIMA-1 met (APR-246) PK11007, and

COTI-2, have been found to exhibit anticancer activity in preclinical BC models, and clinical trials are guaranteed [24].

PI3K/AKT/mTOR Signaling Pathway

Despite the low mutation rate in the PIK3CA gene (5–13%) [23, 25], the PI3K/AKT/mTOR (phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin)-dependent pathway is one of the most important pathways associated with TNBC. PTEN (phosphatase and tensin homologue), INPP4B (inositol polyphosphate 4-phosphatase type II), and other phosphatases are key regulators of this pathway [26]. Mutated or lost PTEN occurs in 35–50% of cases and INPP4B loss in 30% [27]. AKT activation is a potential predictive biomarker to AKT inhibitors being pAKT an activity marker [27] with a low mutation rate; MAGI3-AKT3 translocation occurs in 7% of TNBC and led to constitutive AKT3 activation [28]. LOTUS and PAKT phase II trials have suggested that this pathway may be successfully targeted. The addition of an AKT inhibitor to paclitaxel as first-line therapy for metastatic TNBC improved progression-free survival (PFS) over placebo (stratified HR 0.59; 95% CI: 0.26–1.32, $p = 0.018$) in patients who harbored some alterations in this pathway [29]. Similar findings have been published recently with capivasertib (HR, 0.30; 95% CI, 0.11–0.79; 2-sided $p = 0.01$) [30]. Phase III trials with AKT inhibitors in TNBC are ongoing (NCT03997123, NCT03337724, and NCT04177108).

BRCA

Approximately 20% of TNBC had a BRCA1/2 mutation (12% germline and 8% somatic). Additionally, homologous recombination deficiency as well as genetic and epigenetic inactivation of other components can occur in sporadic cancers (PALB2, BARD1, BRIP1, RAD51B, RAD51C, RAD51D, ATM, FAAP20, CHEK2, FAN1, FANCE, FANCM, and POLQ), defined as “BRCAness” condition [31]. BRCA1/2 mutations and “BRCAness” condition determine a greater sensitivity to platinum chemotherapy as well as to inhibitors of the DNA repair enzyme poly-ADP ribose polymerase 1 (PARP). Both talazoparib and olaparib are indicated in HER2-negative metastatic BC with BRCA1/2 germinal mutations based on 2 randomized phase 3 trials [32, 33]. In a small neoadjuvant trial for germline BRCA1/2 mutated and localized BC, talazoparib achieves 53% pCR rate and residual cancer burden 0 or 1 in 63% of them [34].

Copy Number Variation

In a 465 primary TNBC cohort, MYC was the most frequently affected gene by somatic copy number variation (gained in 81% of patient samples), with frequent

gains in E2F3 (55%), CCNE1 (47%), EGFR (47%), CCND1 (44%), and MYB (41%), and frequent losses in CHD1 (lost in 71% of samples), PTEN (58%), RB1 (54%), and CDKN2A (43%), similarly to the TCGA cohort [22, 35].

DNA Methylation

Finally, DNA methylation is another strong biomarker for TNBC. The TCGA identified 5 distinct DNA methylation groups. Group 5 showed the lowest levels of DNA methylation, overlapped with the basal-like mRNA subtype, and showed a high frequency of TP53 mutations. BRCA1 promoter methylation was associated with poorer OS and recurrence-free survival in TNBC, and is under evaluation as a predictive response biomarker of PARP inhibitors in clinical trials [36]. Using whole-genome DNA methylation analysis, we can divide TNBC into 3 prognostic subgroups, correlate DNA methylation with TNBC gene expression, and predict higher pCR rates after NACT [37, 38].

Molecular BC Classification Based on mRNA

Analysis of gene expression profiles from BC was carried out by Perou et al. [39] and classified tumors into 5 clusters, called intrinsic subtypes: luminal-A, luminal-B, HER2-enriched, basal-like, and a normal-like BC group. These entities show significant differences in incidence, survival, and response to therapy [40]. Although both clinical and biological features distinguish the 5 intrinsic subtypes, relevant variation exists within each group [41]. Additional gene expression analyses have demonstrated the presence of a new intrinsic subtype, claudin low, representing 7–14% of all BCs [42]. Clinically, the majority of claudin-low tumors are TNBC (70%), with a high frequency of metaplastic and medullary differentiation. Although claudin-low and basal-like subtypes share low luminal and HER2 gene expression, claudin-low tumors also show low expression of proliferation-associated genes, in contrast to basal-like subtype.

Molecular TNBC Subtypes

TNBC is not a single entity but a very heterogeneous disease that can be classified into different molecular subtypes by gene expression profiling. The first effort to molecularly classify TNBC subtypes was a direct comparison of 374 TNBC samples extracted from 14 data sets where investigators tried to establish the relationship between the PAM50 intrinsic subtypes [39] and TNBC molecular subtypes [43]. The majority of the TNBC samples were classified by PAM50 assay as basal like (80.6%), followed by the HER2-enriched intrinsic subtype (10.2%), normal-like (4.6%), luminal-B (3.5%), and luminal-A subtypes (1.1%; Table 1) [44].

Table 1. BC intrinsic subtypes and TNBC subtypes based on gene expression profiles

BC intrinsic subtypes Perou et al. [39]	TNBC type Lehman et al. [45]	TNBC type 4 Lehman et al. [47]	TNBC subtypes Burstein et al. [48]
Basal like	Basal like 1 (BL1)	Basal like 1 (BL1)	Basal like, immune suppressed (BLIS)
Her2 enriched	Basal like 2 (BL2)	Basal like 2 (BL2)	
Luminal A	Immunomodulatory (IM)		Basal like, immune activated (BLIA)
Luminal B	Mesenchymal (M)		
Claudin low	Mesenchymal stem like (MSL)	Mesenchymal (M)	Mesenchymal (MES)
Normal like	Luminal androgen receptor (LAR)	Luminal androgen receptor (LAR)	Luminal androgen receptor (LAR)
	Unstable (UNS)		

The emergence of gene expression profiles allowed Lehman et al. [45] to divide TNBC into 6 distinct subtypes: basal-like 1 and 2 (BL1 and BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM), and luminal androgen receptor (LAR). Then, an unstable subtype was also included [45]. These subtypes are characterized by different patterns of molecular alterations in terms of RNA expression, somatic mutations, and copy number variation that tend to cluster in genes involved in specific pathways. After neoadjuvant therapy of early TNBC tumors, the BL1 subtype had the highest pCR rate (52%), while BL2 and LAR had the lowest (0 and 10%, respectively). Lehman subtypes seem to be able to predict pCR probability better than PAM50 intrinsic subtypes [46].

Using more advanced laboratory techniques, Lehmann et al. [47] have subsequently demonstrated that the presence of stromal cells in tumor specimens (TILs and tumor-associated mesenchymal cells) affects the definition of the IM and MSL subtypes, respectively. This led to a new classification, TNBC type 4, including 4 stable transcriptional subtypes (BL1, BL2, M, and LAR). Additionally, the 4 TNBC subtypes showed significant differences in pCR rate when treated with standard NACT, with 46% of BL1 patients reaching pCR, compared to 12, 29, and 15% for patients with BL2, M, and LAR subtypes, respectively ($p = 0.04$). In the meantime, Burstein et al. [48] described stable molecular TNBC phenotypes using gene expression profiling. They distinguish 4 subtypes: LAR, mesenchymal (MES), basal-like, immune suppressed (BLIS), and basal-like, immune activated (BLIA). Interestingly, the BLIS subtype showed the worst prognosis, and the BLIA subgroup conferred the best outcome in terms of DFS.

TNBC Subtypes according to Molecular Profiling

The BL1 subtype is heavily enriched in genes involved in DNA damage response (ATR/BRCA) and cell division pathways, including the highest rate of TP53 mutations (92%), high gain/amplifications of MYC and CDK6, and deletions in BRCA2, PTEN, MDM2, and RB1 [49]. The highly proliferative nature of this entity is further associated

with high Ki-67 mRNA expression. Enrichment of proliferation genes and increased Ki-67 expression in basal-like TNBC tumors suggest that antimitotic agents such as taxanes could be a useful therapeutic strategy [50]. The BL2 subtype involves high levels of growth factor signaling and metabolic pathways (EGF, NGF, MET, Wnt/ β -catenin, and IGF1R). Genes related with immune cell processes, such as B- and T-cell receptor signaling, antigen presentation, and cytokine pathways (JAK/STAT, TNF, and NF κ B) are highly expressed in the IM subtype. Both mesenchymal TNBC subtypes and MSL share high expression of genes related to the epithelial-mesenchymal transition and growth factor pathways. However, only the MSL subtype has low expression of proliferation genes. This decreased proliferation is attached to high expression of genes associated with stem cells. The MSL subtype also displays low expression of claudins 3, 4, and 7, consistent with the claudin-low subtype of BC [42]. Finally, the LAR subtype is the most different among the TNBC subtypes. Despite ER negativity, these tumors are enriched in hormonally regulated pathways (FOXA1 and GATA3) and mRNA and proteins of AR. Thus, not surprisingly, LAR tumors frequently present mutations in PIK3CA (55%), KMT2C (19%), CDH1 (13%), NF1 (13%), and AKT1 (13%) [45].

Immune Characterization and Immuno-Oncology Biomarkers in TNBC

Immunotherapy, and particularly immune checkpoint blockade, is a promising therapy that has shown to improve outcome in solid malignancies. The poor prognosis and the lack of biomarkers and targeted therapy in TNBC have encouraged basic and translational research in the field of immunotherapy. TNBC is characterized by different immune microenvironments from other subtypes, with a higher density of lymphocytic infiltrates and PD-L1 expression, which may involve clinical and therapeutic impact.

Table 2. Relevant neoadjuvant (a) or adjuvant studies (b) reporting lymphocyte-predominant breast cancer (LPBC) incidence and achieved outcomes

a Relevant neoadjuvant studies

First author, year	Design	Histology	Chemotherapy	LPBC	Tumors with LPBC phenotype	Outcome LPBC	Outcome non-LPBC
Denkert [57], 2010: patients from the GeparDuo trial	Retrospect.	ER-/PR-	Docetaxel DD + doxorubicin vs. doxorubicin + CP → docetaxel	>60%	14.9%	pCR 42.9%	pCR (focal lymphocytes) 19.4% pCR (no lymphocytes) 25%
Issa-Nummer [59], 2013: study PREDICT, from the GeparQuinto trial	Prospect.	Her2-	Epirubicin, CP → taxane ± bevacizumab, everolimus, or lapatinib	>60%	26.2%	pCR 36.6%	pCR 14.3%
Denkert [60], 2015: patients from the GeparSixto trial	Prospect.	TNBC	Paclitaxel + liposomal doxorubicin + bevacizumab ± CBDCA	>60%	28.3%	pCR 74% with CBDCA vs. 43% without CBDCA	pCR 46% with CBDCA vs. 34% without CBDCA
Herrero-Vicent [61], 2017	Retrospect.	TNBC	Anthracycline and taxane	>40%	35.4%	pCR 87% 3-year DFS: 98%	9.4% 3-year DFS: 70%
Denkert [58], 2018: patients from the GeparTrio trial	Retrospect.	HER2-	TAC ×2 → 4 vs. 6 of TAC in responders <i>or</i> TAC ×4 followed by VNR and capecitabine ×4 for nonresponders	>60%	12%	pCR 48.1%	8% (no lymphocytes) – 14% (focal lymphocytes)
O’Loughlin [51], 2018	Prospect.	TNBC	Anthracycline and taxane <i>or</i> anthracycline, taxane, and CBDCA	>50%	12%	pCR 89%	pCR 47%

b Relevant adjuvant studies

Trial	Design	Histology	Chemotherapy	Median TILs	LPBC	Tumors with LPBC phenotype	Outcome (per 10% increase in lymphocyte infiltration)	Other outcomes
Loi [52], 2013: patients from the BIG 02-98 trial	Retrospect.	TNBC	Doxorubicin → CMF <i>or</i> doxorubicin + CP → CMF vs. doxorubicin + docetaxel → CMF <i>or</i> doxorubicin → docetaxel followed by CMF	10% (sTILs) 2% (iTILs)	>50%	10.6%	17% reduction in the risk of relapse 27% reduction in the risk of death	5-year DFS: 92.3% LPBC vs. 61.9% non-LPBC 5-year OS: 92.3% LPBC vs. 71.0% non-LPBC
Loi [53], 2014: patients from the FinHER trial	Retrospect.	TNBC	Docetaxel <i>or</i> VNR ×3 → FEC ×3	–	>50%	14.3%	21% reduction in the risk of distant recurrence 20% reduction in the risk of death	
Adams [54], 2014: patients from ECOG 2197 and ECOG 1199 trials	Retrospect.	TNBC	E2197: doxorubicin + CP vs. docetaxel ×4 E1199: doxorubicin + CP ×4 → different taxane regimens	10% (sTILs) 0% (iTILs)	>50%	4.4%	18% reduction in the risk of distant recurrence 19% reduction in the risk of death	
Pruneri [55], 2016: from the IEO Breast Cancer Database	Retrospect.	TNBC	CMF <i>or</i> CMF + AC	20%	>50%	21.9%	21% reduction in the risk of distant recurrence 21% reduction in the risk of death	10-year DFS (LPBC) 71% 10-year DDFS (LPBC) 84% 10-year OS (LPBC) 96%
Kotoula [56], 2016: patients from 4 prospective trials	Retrospect.	TNBC	Anthracycline – taxane regimens	7% sTILs (all tumors)	>50%	26%	–	7-year DFS: 96.3% (LPBC) vs. 72.8% (non-LPBC) 7-year OS: 100% (LPBC) vs. 76.3% (non-LPBC)

sTILs, stromal tumor-infiltrating lymphocytes; iTIL, intratumoral tumor-infiltrating lymphocytes; DD, dose dense; CP, cyclophosphamide; TAC, docetaxel, doxorubicin, and cyclophosphamide; VNR, vinorelbine; CBDCA, carboplatin; CMF, cyclophosphamide, methotrexate, and fluorouracil; FEC, fluorouracil, epirubicin, and cyclophosphamide; AC, Adriamycin and cyclophosphamide; pCR, pathological complete response; DFS, disease-free survival; DDFS, distant DFS; OS, overall survival.

Tumor-Infiltrating Lymphocytes

TILs are lymphocytes which can be found both surrounding the tumoral cells (stromal TILs) or in intimate contact with tumor cells (intratumoral TILs). Tumors with more than 50% [51–56] to 60% [51, 57–61] lymphocytic infiltrates have been defined as lymphocyte-predominant BC and represent about 20% of TNBC, in contrast to 16% in human HER2+ tumors and 6% in luminal subtypes [62].

The presence of pCR after NACT is strongly correlated with long-term benefit in BC. BC with a high percentage of lymphocytes has significantly demonstrated to be a predictive biomarker for response to NACT in early TNBC, with higher pCR (Table 2a). Regarding residual disease after NACT, higher residual-disease TILs are significantly associated with improved recurrence-free survival and OS [63]. Likewise, in the adjuvant setting, a higher density of TILs is associated with improved DFS, distant DFS and OS (Table 2b). It has been shown that a 10% increase in TILs has a good prognostic value when analyzed by uni- and multivariable adjusted survival analysis for DFS and OS [52–55]. Therefore, it has been established as an independent prognostic factor for TNBC.

The variability in these outcomes probably depends not only on TIL quantity but also on the composition of lymphocytic infiltrates. CD8+ cytotoxic T lymphocytes are essential components of adaptive immunity involved in tumor cell destruction and expressed in 60% of TNBCs [62]. In a pooled analysis of 25 published studies, CD8+ lymphocytes were associated with improved and BC-specific survival [48]. Forkhead box protein 3 (FoxP3+) is a specific molecular marker of CD4+ regulatory T cells (Tregs), found in 70% of TNBCs [62]. The role of FoxP3+ lymphocytes remains controversial since some studies reported improved PFS and OS although Tregs downregulate the antitumor activity of the immune system. It has also been shown that a higher CD8+/FoxP3+ ratio predicts more favorable outcomes in TNBC patients [64].

To standardize TILs evaluating the method in BC, the International TILs Working Group suggests assessing the percentage of TILs from the stromal compartment in hematoxylin- and eosin-stained tumor sections of a needle core biopsy.

Immune Checkpoint Signaling Pathway

The expression and interaction of the immune checkpoint molecules programmed death-1 (PD-1), its ligand PD-L1, and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) are involved in T-cell activity regulation and tumor immune escape mechanisms, and they can be a therapeutic target to immune checkpoint inhibitors.

TNBC have the highest rate of PD-L1 expression, defined as at least 1% of PD-L1-positive cells, compared with other BC subtypes. PD-L1 expression on tumoral and inflammatory cells is significantly correlated with

stromal TIL levels [65, 66], thus high PD-L1 levels have also been associated with an increased probability to achieve pCR after NACT and specific survival in TNBC [67]. Even though, recently, the Keynote-522 trial revealed that the addition of pembrolizumab to chemotherapy in the neoadjuvant setting significantly increased pCR and DFS both in PD-L1-positive or -negative subgroups [68]. Some authors found that patients with high PD-L1 expression and low levels of stromal TILs have the most unfavorable prognosis [65, 69], and probably those patients will benefit most from immunotherapy agents.

In the metastatic setting, PD-L1 has shown to be a clinical biomarker for the use of anti-PD-L1 agents like atezolizumab, with a notable benefit in terms of objective response rate, PFS, and OS (IMpassion-130 trial [70]). For pembrolizumab, data from the Keynote-086 study [71] suggest a modest benefit in the disease control rate for PD-L1 patients, with a long-lasting subset of patients with PD-L1-positive tumors. Combination therapy is currently under research, and the latest data have shown that combination chemotherapy with pembrolizumab increases PFS compared to chemotherapy only in patients with metastatic TNBC and PD-L1-positive tumors [72].

PD-L1 testing variability may depend on several factors, such as interpathologist variability or the use of different IHC assays. Studies conducted to standardize PD-L1 testing in the clinical setting demonstrated concordant results using the 22C3, SP263, and 28-8 anti-PD-L1 antibodies in BC [73] and other tumors [74]; however, the SP142 assay seems to underestimate high PD-L1 positivity in approximately 20% of the tumors. In the same way, PD-L1 positivity can also be defined by different scoring systems: the percent tumor area covered by PD-L1-positive immune cells (IC score), assessed in atezolizumab trials, or the ratio of PD-L1-positive cells out of the total number of tumor cells (combined positive score), approved for pembrolizumab.

Tumor Mutational Burden

The presence of tumor antigenic epitopes, sprung from mutations in tumor cells, induces T-cell immune responses. BC is characterized by lower mutational loads than other tumors, but it varies among BC subtypes, with the highest average TMB for TNBC, followed by HER2+, and the lowest average for ER+. TMB-Hi tumors with a favorable immune-infiltrate disposition have been associated with a prolonged survival trend, but the statistical significance has not been achieved [75]. Further studies are needed to contrast this outcome.

Immune Gene Expression Signature

Several immune-related gene expression signatures have been described to typify the tumor immunogenicity

Table 3. Immune gene signatures in triple-negative breast cancer (TNBC)

Gene signatures	Gene lists	Association with clinical outcome
7-gene expression signature [76]	7 genes related to immune/defense response (<i>C1QA</i> , <i>IGLC2</i> , <i>LY9</i> , <i>TNFRSF17</i> , <i>SPP1</i> , <i>XCL2</i> , <i>HLA-F</i>)	High immune response module → favorable prognosis in ER– BC
14-gene HR–/TNBC prognostic signature index [77]	14 prognostic genes (<i>CXCL13</i> , <i>CLIC5</i> , <i>RGS4</i> , <i>RPS28</i> , <i>RFX7</i> , <i>EXOC7</i> , <i>HAPLN1</i> , <i>ZNF3</i> , <i>SSX3</i> , <i>HRBL</i> , <i>PRRG3</i> , <i>ABO</i> , <i>PRTN3</i> , <i>MATN1</i>) including 8 genes linked to immune/inflammatory chemokine regulation	High HR–/TNBC index → poor prognosis in HR– BC
Ratio of high B cell and low IL-8 metagenes [78]	B-cell metagene including IgG IL-8 metagene including <i>IL-8</i> , <i>CXCL1</i> , <i>CXCL2</i>	High B-cell/low IL-8 metagenes → favorable prognosis in TNBC
Optimized 5-gene predictor (integrated cytokine score) [79]	Interferon γ - and IL-10-related pathway (<i>TNFRSF17</i> , <i>CLIC5</i> , <i>HLA-F</i> , <i>CXCL13</i> , <i>XCL2</i>)	High integrated cytokine score → poor prognosis in HR– TNBC
4-gene signature [80]	Expression levels of 4 genes (<i>HLF</i> , <i>CXCL13</i> , <i>SULT1E1</i> , <i>GBP1</i>) predict the extent of lymphocytic infiltration after NACT	High TILs after NACT → increase distant relapse-free survival

BC, breast cancer; ER, estrogen receptor; HR, hormone receptor; NACT, neoadjuvant chemotherapy; TILs, tumor-infiltrating lymphocytes.

in different cancer types, including TNBC (Table 3) [76–80]. In TNBC, a 4-gene signature including *HLF*, *CXCL13*, *SULT1E1*, and *GBP1* showed correlation with the presence of high TILs after NACT and increased distant relapse-free survival in a multivariate analysis (HR 0.29, 95% CI 0.13–0.67) [80]. Another 7-gene module including *C1QA*, *IGLC2*, *LY9*, *TNFRSF17*, *SPP1*, *XCL2*, and *HLA-F* showed immune response pathway activation and better prognosis in an ER-negative BC cohort [76]. Although available data strongly suggest that immune gene signature will be a useful approach for defining the immune characteristics of tumors, future research should be conducted to define a unified gene expression profile that can be implemented in clinical practice.

Conclusion

This review highlights the inherent problem related with the TNBC definition, as it does not reflect the complexity of this disease including intrinsic molecular and immunological heterogeneity, and a huge variety of clinical phenotypes. In the near future, a better comprehensive subclassification of TNBC incorporating new and

complete panels of biomarkers and immune-molecular signatures should be considered for the design of future clinical trials in early and advanced TNBC.

Acknowledgment

We would like to thank Breast Care and Dr. Saura for the opportunity to perform this review focused on TNBC.

Disclosure Statement

Dr. Ciruelos reports personal fees from Roche, Lilly, Novartis, and Pfizer. The other authors have no conflicts of interest to declare.

Funding Sources

The author(s) received no specific funding for this work.

Author Contributions

All authors contributed equally to this manuscript.

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